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Cytometry Part A

Disclaimer

Monitoring Dynamic Protein Expression in Living *E. Coli.* Bacterial Cells by Laser Tweezers Raman Spectroscopy[1]

James W. Chan[1,3], Heiko Winhold[1], Michele H. Corzett[2], Joshua M. Ulloa[2], Monique Cosman[2,3], Rod Balhorn[2,3], and Thomas Huser[3,4]

[1] Applied Physics and Biophysics Division, Physics and Advanced Technologies Directorate, Lawrence Livermore National Laboratory, Livermore, CA

[2]Chemistry, Materials, and Life Sciences, Lawrence Livermore National Laboratory, Livermore, CA

[3]NSF Center for Biophotonics Science and Technology, University of California, Davis, Sacramento, CA

[4]Department of Internal Medicine, School of Medicine, University of California, Davis, CA

Running headline: Raman Analysis of Dynamic Protein Expression

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Corresponding author: James W. Chan, Physics and Advanced Technologies, Lawrence Livermore National Laboratory, P.O. Box 808, L-211, Livermore, CA 94551, phone: 925-423-3565, fax: 925-424-2778, chan19@llnl.gov

Abstract

Background: Laser tweezers Raman spectroscopy (LTRS) is a novel, nondestructive, and label-free method that can be used to quantitatively measure changes in cellular activity in single living cells. Here, we demonstrate its use to monitor changes in a population of *E. coli cells* that occur during overexpression of a protein, the extracellular domain of myelin oligodendrocyte glycoprotein (MOG(1-120))

Methods: Raman spectra were acquired of individual *E. coli* cells suspended in solution and trapped by a single tightly focused laser beam. Overexpression of MOG(1-120) in transformed *E. coli* Rosetta-Gami (DE3)pLysS cells was induced by addition of isopropyl thiogalactoside (IPTG). Changes in the peak intensities of the Raman spectra from a population of cells were monitored and analyzed over a total duration of three hours. Data was also collected for concentrated purified MOG(1-120) protein in solution, and the spectra compared with that obtained for the MOG(1-120) expressing cells.

Results: Raman spectra of individual, living *E. coli* cells exhibit signatures due to DNA and protein molecular vibrations. Characteristic Raman markers associated with protein vibrations, such as 1257 cm^{-1}, 1340 cm^{-1}, 1453 cm^{-1} and 1660 cm^{-1}, are shown to increase as a function of time following the addition of IPTG. Comparison of these spectra and the spectra of purified MOG protein indicates that the changes are predominantly due to the induction of MOG protein expression. Protein expression was found to occur mostly within the second hour, with a 470% increase relative to the protein expressed in the first hour. A 230% relative increase between the second and third hour indicates that protein expression begins to level off within the third hour.

Conclusion: It is demonstrated that LTRS has sufficient sensitivity for real-time, nondestructive, and quantitative monitoring of biological processes, such as protein expression, in single living cells. Such capabilities, which are not currently available in flow cytometry, open up new possibilities for analyzing cellular processes occurring in single microbial and eukaryotic cells.

Key terms: Raman spectroscopy, optical tweezers, laser trapping, protein expression, glycoprotein, E. coli, single cells

Introduction

Many cells interact with their environment as independent entities and subtle differences in their responses can have dramatic consequences that relate to cancer induction and embryogenesis. Thus, performing noninvasive chemical analysis and monitoring long-term dynamic changes in metabolic processes of single living cells are capabilities that have been long sought after. Since individual cells may act spontaneously and can behave differently upon activation, it is often useful to obtain information from individual cells and determine distributions rather than only an averaged response. Acquiring real-time information of live single cell dynamics is also valuable for obtaining a better understanding of the biological response among a heterogeneous population of different cells. Current techniques, such as gel electrophoresis, blotting, mass spectrometry, and reverse transcription polymerase chain reaction (RT-PCR) lead to averaged biochemical data from many cells, whereas fluorescence microscopy and flow cytometry require monoclonal antibodies and exogenous fluorescent labels. The aim of this current work is to demonstrate the unique abilities of a novel laser based method known as laser tweezers Raman spectroscopy (LTRS) to monitor real-time biochemical changes of a single living cell upon induction of protein expression.

Raman spectroscopy is a powerful analytical tool that is based on the inelastic scattering of photons by molecular bonds. Photons that scatter off of a molecular bond and excite the bond to a higher vibrational state are known as Stokes-shifted Raman photons, and are registered as distinct red-shifted peaks in a Raman spectrum. Raman spectra are highly specific to the composition or chemical and physical state of a cell, and changes in these properties are reflected by peak shifts or intensity changes in the spectra. One of the main benefits of Raman spectroscopy

is that it is laser based, making it a noninvasive approach to interrogate single, living cells, provided that lower energy photons at moderate powers are used to minimize overall absorption by the cells[1]. More importantly for the work discussed here is that Raman spectroscopy possesses the level of sensitivity required to follow biochemical changes inside individual, living cells.

Several recent studies[1-4] have demonstrated the benefit of combining optical trapping (i.e. laser tweezers) with confocal Raman spectroscopy for the analysis of micron-sized particles in solution. A single tightly focused laser beam can function as both a single beam optical trap to suspend micron-sized transparent particles near the laser focus and as the excitation source to obtain their Raman spectra in a highly efficient manner. When applied to living cells and microorganisms, this approach presents a method to rapidly analyze the biochemical composition of individual living cells in suspension. For example, LTRS has been applied to the rapid analysis of mixed populations of bacterial spores and bacteria[2,5,6]. It has been applied in the medical field to the analysis of lipoproteins[7], synaptosomes[8], and individual cancer cells[9,10]. In combination with micro-fluidic cell delivery, LTRS can also be used for label-free cell sorting[6]. To a lesser extent, monitoring single cell biological processes with LTRS in real time, such as heat-denaturation of microorganisms[11], the kinetic germination of bacterial spores[12], and the biological state of cells[4,13,14], has also been demonstrated.

Here, we focused on demonstrating the capability of LTRS to monitor the induction of protein expression in individual *E. coli* cells transformed with the gene for the extracellular domain of myelin oligodendrocyte glycoprotein (MOG(1-120)). MOG is a 26 kD to 28 kD integral membrane protein of the central nervous system implicated as a target for auto aggressive antibodies in multiple sclerosis[15]. MOG (1-

120) protein was chosen for our study because of its high expression level in *E. coli* cells. Activation-dependent changes in the level of MOG protein expression can be followed in real time and at the single cell level by analyzing the Raman spectra of individual *E. coli* cells.

MATERIALS AND METHODS

Confocal Raman Microscope

A schematic of the optical setup is depicted in Figure 1. The laser beam of a 30 mW helium-neon (He-Ne) laser (Spectra Physics) at 633 nm is directed through a bandpass filter (Omega Filters) to remove undesired plasma emissions from the laser beam. A telescope expands the beam diameter to roughly 6 mm to match the diameter of the input aperture of the microscope objective. The laser beam is introduced into an inverted optical microscope (Axiovert 200, Zeiss) equipped with a dichroic mirror and a 100x, 1.3 NA oil immersion objective (Plan-NEOFLUAR, Zeiss). The beam forms an optical trap at the laser focus and the Raman signals generated at the laser focus from a trapped cell are epi-detected, spatially filtered by a 100 μm diameter confocal pinhole, and sent through a 633 nm holographic notch filter (Kaiser Optical) for the rejection of the Rayleigh scattered light. The Raman signals are directed into a spectrometer (Triax 320, Jobin-Yvon) equipped with a 1200 grooves/mm grating blazed at 500nm and a liquid nitrogen cooled 1340 x 100 pixel back-illuminated CCD camera (Roper Scientific). Data is acquired using Winspec (Roper Scientific) computer software. Images of trapped cells are observed under white light illumination and imaged with a second CCD camera attached to the microscope.

Protein Expression and Purification

The nonglycosylated, extracellular domain of rat myelin oligodendrocyte glycoprotein (MOG(1-120)) has been previously cloned into a pET32 vector that codes for residues 1-120 with an additional Met residue at the N-Terminus derived from the vector and a LEHHHHHH-tag at the C-terminus for facilitating the purification. Expression levels of aproximately 10-15 mg/L of MOG(1-120) in Luria-

Bertani (LB) broth are typically obtained in an *E. coli* Rosetta-Gami(DE3)pLysS host (Novagen). The expression *E. coli* host Rosetta-Gami(DE3)pLysS (Novagen) was used for its enhanced cytoplasmic disulfide bond formation and enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli*.

E. coli culture was diluted 1 to 100 into LB media containing100 μg/ml ampicillin and 34 μg/ml chloramphenicol and grown at 37 °C until the A_{600} value was 0.6-0.8 (4-5 hours). Protein expression was induced by addition of IPTG to a final concentration of 1.0 mM. The cultures were grown for >5 hours at 37 °C and harvested by centrifugation (2600 G for 20 minutes at 4 °C).

Bacteria pellets containing MOG(1-120) were resuspended in sonication buffer (100 mM Tris-HCl, pH 8.0, 1M NaCl, 10 mM imidazole) with added protease inhibitors (1 Roche Complete, EDTA-free; Protease Inhibitor Cocktail Tablet/50 ml and Phenylmethanesulfonyl fluoride (PMSF) to 1 mM final concentration) . Cells were lysed by sonication 4 times, each for 10 seconds with a 10 minute interval between sonications, after which the insoluble cellular debris was pelleted by centrifugation (20,000 G for 30 minutes at 4 ^{o}C). The resulting supernatant was bound to Ni-NTA-agarose column (Qiagen) by incubating 2 hours at 4 ^{o}C on an orbital rocker. The protein-agarose matrix was then loaded into a disposable 10 ml column (Qiagen) and washed with two column volumes of 100 mM Tris-HCl, pH 8.0, 1M NaCl, 10 mM imidazole, followed by a wash with the same buffer containing 30 mM imidizole instead of 10 mM. MOG was eluted off the column using the same buffer containing 90 mM imidizole and collected in 10, 2 ml fractions. The purity of MOG(1-120) protein in each fraction was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) with a major band at the expected

molecular weight (14.4 kD) and a very minor band at ~32 kD corresponding to a dimer. The quantity of protein was determined by the Bradford method.

Raman Experiments

An initial experiment was performed to determine the spectral changes of *E. coli* cells before and after exposure to IPTG. Approximately 2 μL of an *E. coli* sample taken directly from the shaker was diluted with 100 μL of phosphate buffered saline (PBS) solution to ensure that only single cells are trapped without interference from neighboring cells. The samples were placed onto a microscope coverslip for Raman analysis. The time for acquiring each spectrum is typically 60-120 seconds per cell.

Real-time monitoring of IPTG-induced protein expression of *E. coli* cells directly on the microscope were carried out using a glass bottom culture dish (DH3522, WillCo Wells) to hold the samples. A 3 mL PBS solution containing *E. coli* cells is used in these measurements. The large volume of liquid ensures that the solution will not dry out during the three hour experiment. The metabolic activity of the cells is sustained in PBS solution for the duration of the experiment, but the bacteria are kept at near starving conditions. The solution in the culture dish is heated to 37°C with a micro incubation chamber (DH-35i, Warner Instruments) connected to a temperature controller (TC-344B, Warner Instruments) that maintains the temperature within 0.1°C from the set temperature. The micro incubation chamber is placed on the inverted microscope stage and an individual *E. coli* bacterium is optically trapped in the culture dish. Typical laser powers used for optical trapping and Raman acquisition are ~10 mW. At time t=0, IPTG is added to the culture dish solution to a final IPTG concentration of 1 mM. Individual cells are trapped and Raman spectra are acquired every 2 minutes during the induction process for a total of 3 hours.

A spectrum of purified MOG(1-120) protein dissolved in PBS was recorded to determine its characteristic Raman markers, which can then be compared to changes observed in the induced *E. coli* spectra. The Raman spectra of MOG(1-120) was obtained by focusing the laser beam into a 1 mM solution of the protein. It should be noted that at this concentration, the protein begins to aggregate in solution, so the laser beam was directed onto these regions identified by white light imaging on the CCD camera.

All spectra were background subtracted by fitting the broad background to a third-order polynomial to remove undesired auto-fluorescence and background contributions. The spectra were normalized to the 1095 cm^{-1} peak, which is associated with the DNA backbone ($O\text{-}P\text{-}O^-$) vibration. This peak is chosen because its intensity remains constant during the entire experiment, since the DNA content is not expected to change during MOG(1-120) expression.

Results

A comparison of the Raman spectra acquired for un-induced cells and cells incubated with IPTG is shown in Figure 2. Each spectrum consists of an average of 20 different individual cells and has been background corrected and normalized. The data shows that the intensity of several Raman peaks located at 1257 cm^{-1}, 1340 cm^{-1}, 1453 cm^{-1} and 1660 cm^{-1} increase significantly following induction of the *E. coli* cells. Other peaks such as the 785cm^{-1} and 1001cm^{-1} peak show no noticeable changes in intensity or shifts. The assignments of the Raman peaks found in the *E. coli* spectra are shown in Table 1. They are consistent with previously published results from work using Raman spectroscopy to characterize *E. coli* bacteria[11].

A background-corrected Raman spectrum of MOG(1-120) protein in PBS solution is shown in Figure 3. Several characteristic peaks at 1004 cm^{-1}, 1257 cm^{-1}, 1340 cm^{-1}, 1453 cm^{-1} and 1660 cm^{-1} are observed, suggesting that the increase in the Raman peaks at these wavelengths in the spectra of the IPTG-induced *E. coli* cells are due to the expression of this protein.

The evolution of the spectral changes in *E. coli* cells exposed to IPTG monitored over a 3 hour time period is depicted in Figure 4a. The data indicate that the 1257 cm^{-1}, 1340 cm^{-1}, 1453 cm^{-1} and 1660 cm^{-1} peaks all increase during the 3 hours the cells are exposed to IPTG, with no noticeable changes in the other Raman peaks. Results from control experiments that monitor an uninduced cell over the same time frame indicate that there are no noticeable changes in the Raman spectra, as shown in Figure 4b. For example, the intensity change of the 1340 and 1453 cm^{-1} peaks after 3 hours is 0.043 and 0.017, which is considered to be negligible compared to the changes following induction.

Each spectrum at a given time point in Figure 4a comprises an average of 5 spectra of 5 different cells. Since the acquisition time for a single cell spectrum is 2 min., each averaged spectrum represents the changes in the cells that occur over a 10 min. time frame. The reason for conducting the experiment in this manner is to avoid any potential issues with laser damage from the optical trap. We have observed that trapping of a single cell for greater than 10 minutes induces spectral changes, which is depicted in Figure 5. The most noticeable changes are the decrease in intensities of several peaks, including the 647, 785, 1257, 1340, 1578, and 1660 cm^{-1} peaks. These Raman markers are associated with both DNA and proteins.

Discussion

Germane to the current study are the peaks located at 1257 cm^{-1}, 1340 cm^{-1}, 1453 cm^{-1} and 1660 cm^{-1}, which are all general Raman markers characteristic of proteins and are observed in the Raman spectra of MOG(1-120). These peaks are also shown to increase in intensity in the spectra of IPTG-induced *E. coli* cells. The 1257cm^{-1} and 1660cm^{-1} peaks are assigned to the amide III and amide I vibrations, the 1340cm^{-1} peak is attributed to a protein C-H deformation, and the 1453cm^{-1} peak is due to CH_2 protein deformation.

The increase in peak intensities of these protein Raman modes for *E. coli* cells incubated with IPTG (Figure 2) confirms that these cells undergo a change of their intracellular protein concentration once protein expression is induced. We attribute these changes to the specific over-expression of the MOG(1-120) protein, which is confirmed by SDS-PAGE (Figure 6). The spectral changes are consistent and overlap with the Raman peaks obtained from the purified MOG(1-120) protein solution (Figure 3), which further indicate that MOG(1-120) protein expression is predominately the cause of the spectral changes.

In addition, no changes were observed in the DNA peaks at 783 cm^{-1} and 1578 cm^{-1}. The constant intensity of the DNA peaks is consistent with the fact that induced cells do not exhibit growth and division and are primarily active in expressing the MOG(1-120) protein. This result also indicates that there is no *spectral* evidence of major damage to the cells as a result of laser exposure within the 2 minute acquisition time, since a previous study[16] had reported that laser damaged cells exhibit a decrease in DNA peak intensity at 1578 cm^{-1}. We have also observed similar spectral changes associated with DNA and protein Raman markers (Figure 5) as a result of the harmful effects of long-term laser exposure to the cells. In order to

avoid this deleterious effect in our experiments, different individual cells were probed for short durations (2 min.) in order to decrease the potential of laser damage. An average of these spectra was taken to yield the results shown in Figure 4a. An alternative method to reduce laser damage, as demonstrated by Xie *et. al.*[1], would be the use of longer wavelength light (e.g. at 785 nm) and a power switching scheme, where the laser power is switched from low to high for cell trapping and Raman acquisition, respectively.

The results of these experiments show that real time measurements of the changes occurring in the induced *E. coli* cells can be monitored using the laser trapping Raman method (Figure 4). There is minimal change in the Raman spectra within the first hour (data not shown), indicating minimal expression of the protein immediately after induction. Significant increases in protein expression seem to occur only within the second hour and continue into the third hour after induction. The intensity changes for the 1257 cm^{-1}, 1340 cm^{-1}, 1453 cm^{-1} and 1660 cm^{-1} peaks are shown in Figure 6. The relative peak intensities of these peaks compared to the 1257 cm^{-1} amide III protein peak after induction are 1, 1.34, 2.17, and 1.83, respectively. These ratios are similar to those obtained for the purified MOG(1-120) protein in Figure 3 (1, 1.33, 1.95, 1.75, relative to the 1257 cm^{-1} peak), which strongly suggest that the changes in the *E. coli* spectra are indeed due to MOG(1-120) over-expression. We estimate from the peak intensity values that the rate of protein expression is highest within the second hour, with a 470% increase in protein concentration when comparing the signal intensity at the one and two hour time points. The expression continues in the third hour, but the rate begins to level off, as evidenced by a 230% increase in protein levels between the second and third hour. To confirm that the change is not a response of the cells being kept at near starving

conditions in PBS solution, equivalent experiments were performed where cells were probed over a 3 hour time frame without the addition of IPTG. No changes in the DNA or protein Raman markers were observed, indicating that the cells remained viable for this time frame, and that the changes in the protein markers shown in Figures 5 and 6 are, in fact, associated with IPTG induction of the MOG protein expression.

Conclusion

We demonstrate the unique capabilities of LTRS to obtain label-free chemical information from individual living cells and monitor the temporal evolution of a biological process by using the IPTG-induced overexpression of the MOG(1-120) protein in single transformed *E. coli* cells as a model system. We have shown that it is possible to use this method to non-invasively and quantitatively monitor protein expression at the single cell level over an extended period of time. Increases in Raman protein markers were clearly observed after induction of protein expression, which allowed a relative increase in protein concentration to be determined. This work sets the foundation for a number of additional experiments that are planned to follow biochemical changes in living microbial cells in real-time due to changes in their environment, e.g. gene expression after transitions from anaerobic to aerobic states. The current results also suggest that it should be possible to investigate changes induced in individual microbial cells after exposure to environmental hazards. In the future, modification of the LTRS system to utilize longer wavelength excitation light will further help to reduce laser-induced damage and enable a single cell to be monitored continuously for the entire 3 hours.

Tables, Figures, and their Legends

Fig. 1 Schematic of a confocal LTRS setup. A He-Ne laser is focused into a sample with a high NA microscope objective. Raman signals are epi-detected, spatially filtered by a confocal pinhole and dispersed onto a liquid nitrogen cooled CCD camera attached to a spectrometer.

Fig. 2 Averaged Raman spectra of *E. coli* bacteria before and after the induction of protein expression. The acquisition time for each spectrum was 120 seconds with an excitation power of 10mW at 633nm. The measurement was repeated for 20 cells and averaged. All spectra are background-subtracted and normalized to the 1095cm^{-1} Raman peak.

Fig. 3 Raman spectrum of purified MOG protein. This spectrum is an average over four spectra and the acquisition time was 240 seconds respectively, with an excitation power of 10mw at 633nm. Each spectrum is obtained from a protein cluster, which is suspended in the laser focus; therefore the whole focal volume is filled with MOG. The relevant peaks are located at 1004cm^{-1}, 1257cm^{-1}, 1340cm^{-1}, 1453cm^{-1} and 1660cm^{-1}.

Fig. 4 (a) Raman spectra of IPTG-induced *E. coli* show dynamic changes from an uninduced to an induced state over a three hour time period. The IPTG concentration in the probe sample is 1mM and the temperature is 37°C. Each spectrum is an average over 5 spectra of different *E. coli* cells. The acquisition time for each spectrum was 120 seconds with an excitation power of 10mW at 633nm. Every spectrum is background-subtracted and normalized to the 1095cm^{-1} DNA backbone peak. For clarity, also shown are the difference spectra at each time point, obtained by subtracting the start spectrum from the spectrum at one, two, and three hours. (b)

Results from a control experiment showing that there is no significant change in the Raman spectra of uninduced cells before and after 3 hours.

Fig. 5. Raman spectra of laser damaged E. coli cells compared with unmodified cells. The modified cells are exposed to the laser beam for 10 minutes. Each spectrum is an average of 5 individual cell spectra. The peaks that change are attributed to DNA and protein vibrations.

Fig. 6 SDS NuPAGE 4-12% gel of whole cell extracts from uninduced and induced for 3hr *E. coli* bacteria. The intense blue band in the induced *E. coli* bacteria is attributed to the expression of MOG protein. For calibration, a standard protein solution with known molecular weights is run simultaneously and shown on the far left of the gel.

Fig. 7 Relative intensity change of the 1257cm^{-1}, 1340cm^{-1}, 1453cm^{-1} and 1660cm^{-1} peaks in the *E. coli* Raman spectra as a function of time after induction of MOG expression.

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but not with 660 nm Laser-Light. *Experimental Cell Research* **195**, 361-367 (1991).

Table 1. Raman peaks of *E. coli* cells and their assignments

Raman frequency (cm^{-1})	Assignment
728	Adenine
783	Nucleic acids (C, T)
813	Tyrosine
857	Tyrosine
936	DNA backbone
1004	Phenylalanine
1095	DNA: $O\text{-}P\text{-}O^-$
1126	p: C-N, C, T
1257	p: amide III
1340	Nucleic acids (A, G)
1453	p: $C\text{-}H_2$ def., lipids
1660	Amide I

Figure 1.

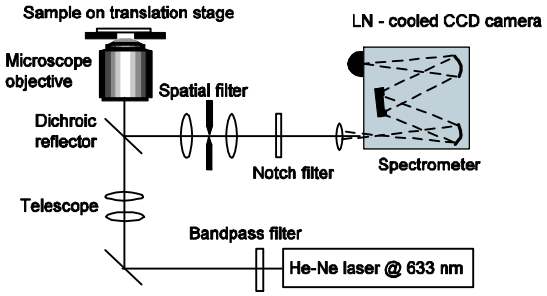

Sample on translation stage
LN - cooled CCD camera
Microscope objective
Spatial filter
Dichroic reflector
Notch filter
Spectrometer
Telescope
Bandpass filter
He-Ne laser @ 633 nm

Figure 2.

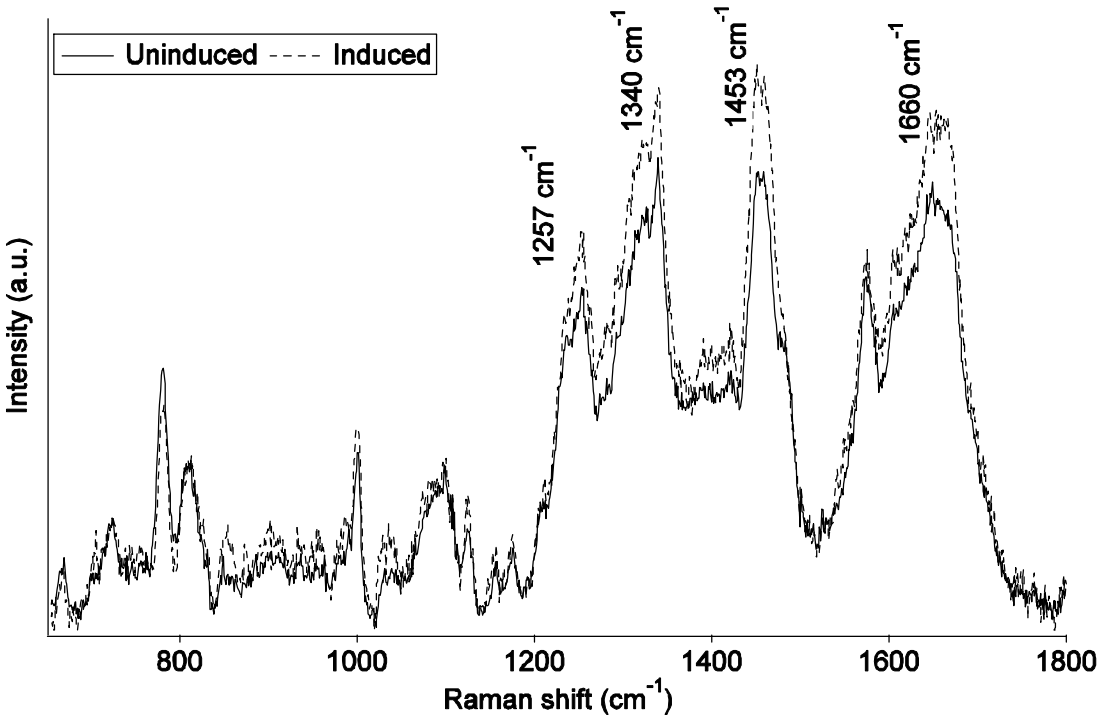

Uninduced
Induced
1257 cm-1
1340 cm-1
1453 cm-1
1660 cm-1
Intensity (a.u.)
800
1000
1200
1400
1600
1800
Raman shift (cm-1)

Figure 3.

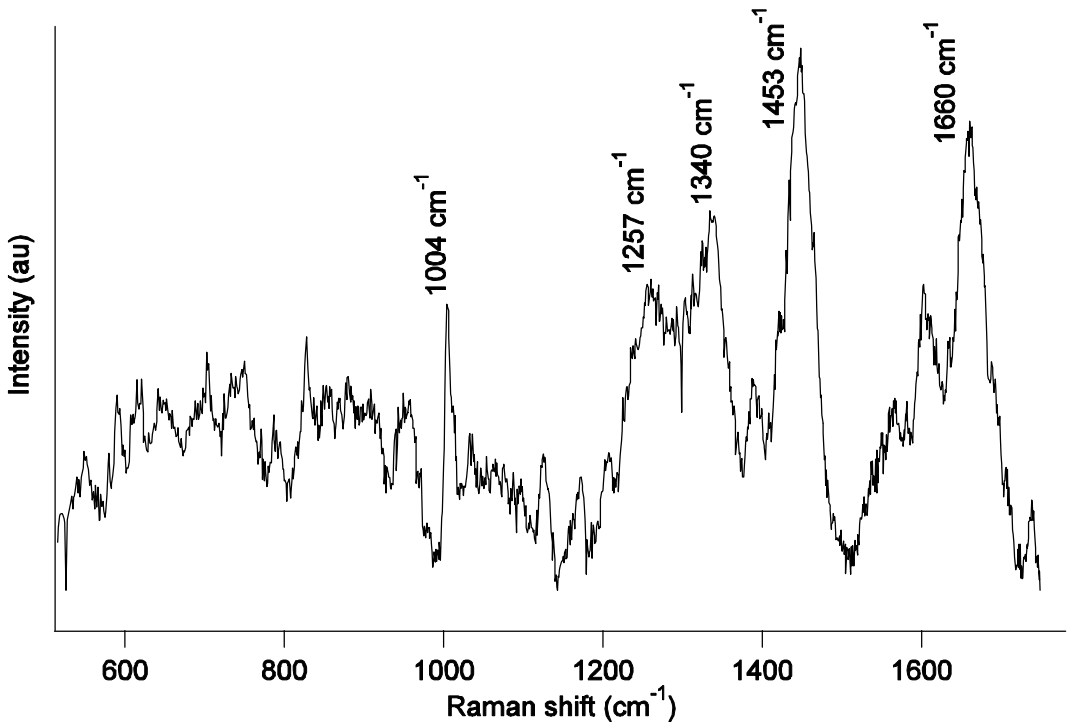

Intensity (au)
1004 cm-1
1257 cm-1
1340 cm-1
1453 cm-1
1660 cm-1
600
800
1000
1200
1400
1600
Raman shift (cm-1)

Figure 4.

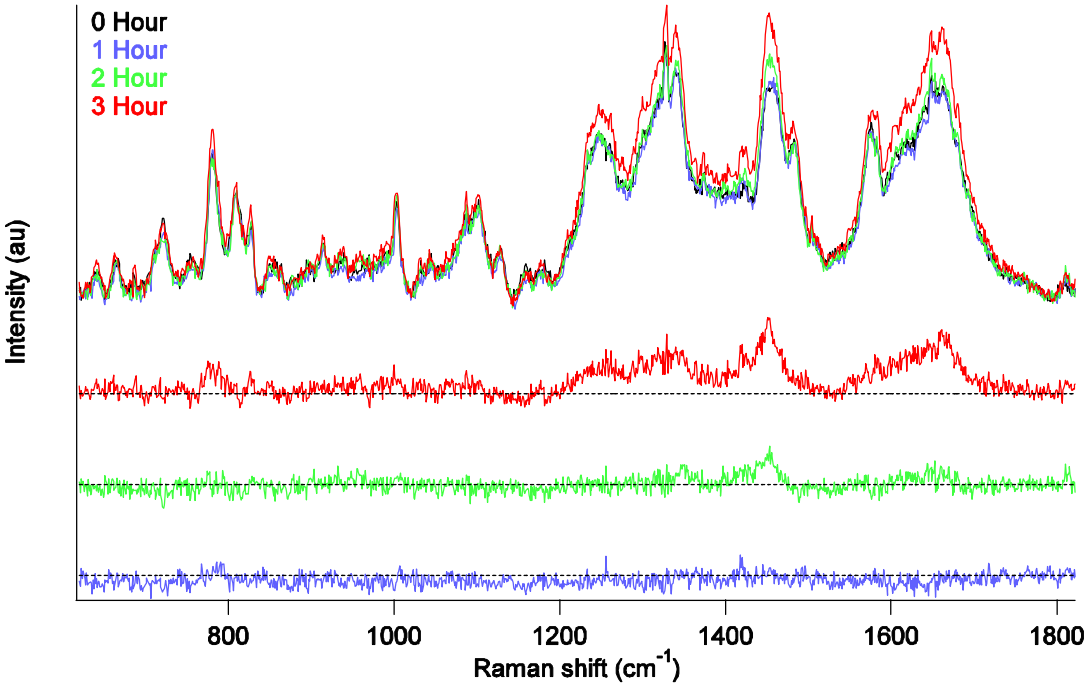

0 Hour
1 Hour
2 Hour
3 Hour
Intensity (au)
800
1000
1200
1400
1600
1800
Raman shift (cm-1)

Figure 5.

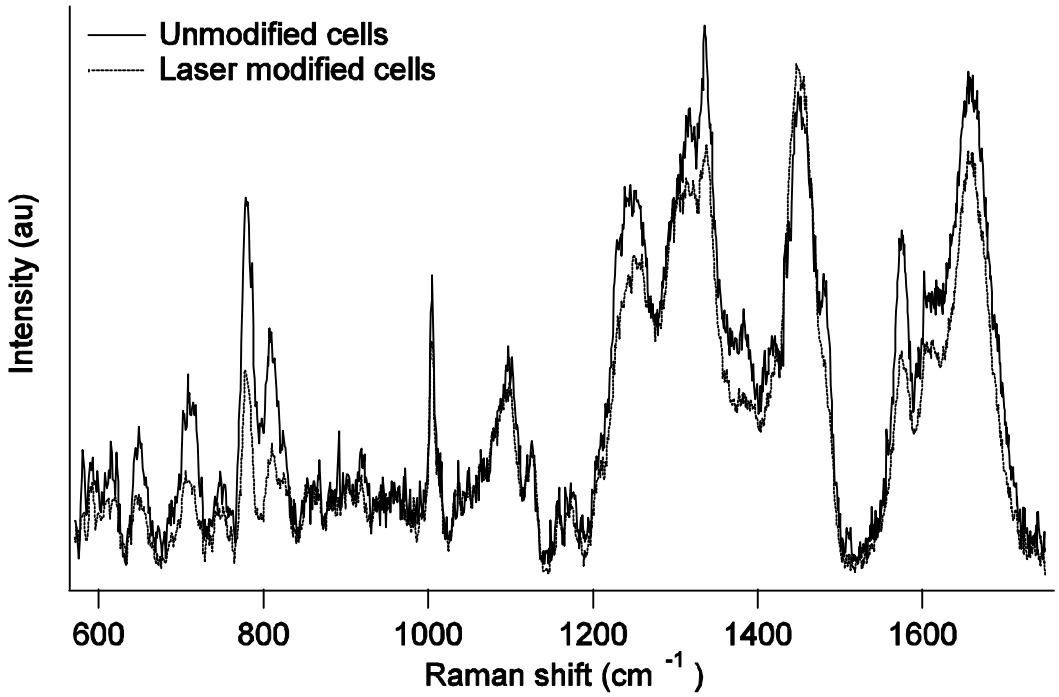
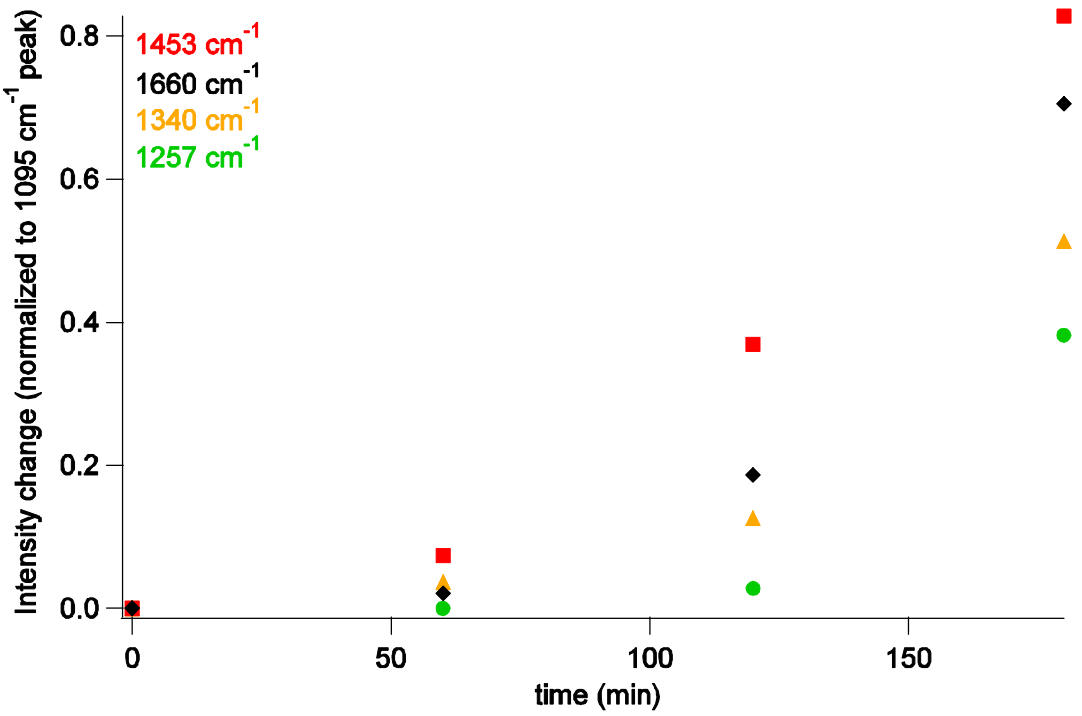

Unmodified cells
Laser modified cells
Intensity (au)
600
800
1000
1200
1400
1600
Raman shift (cm -1)

Figure 6.


1453 cm-1
1660 cm-1
1340 cm-1
1257 cm-1
Intensity change (normalized to 1095 cm-1 peak)
0.0
0.2
0.4
0.6
0.8
0
50
100
150
time (min)